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Transcription factor E4TF1 contains two subunits with different functions

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The transcription factor E4TF1 stimulates transcription from the adenovirus early region 4 promoter by binding to a specific promoter element. E4TF1 has been purified to homogeneity from HeLa cells by sequence-specific DNA affinity chromatography and characterized. E4TF1 is composed of at least two distinct subunits identified as 60 kd and 53 kd polypeptides. The 60 kd protein alone is able to bind to the specific DNA sequence but not to stimulate transcription *in vitro*. The 53 kd protein alone neither binds to DNA nor stimulates transcription *in vitro*. However, the 53 kd protein is able to interact with the 60 kd protein and the interaction confers the ability to stimulate transcription *in vitro* and to increase the DNA binding affinity of the 60 kd protein. This study provides evidence that the interaction between the two different subunits of E4TF1 is required for it to function as a transcription factor, and that one of the subunits binds to a specific DNA sequence and the other works as a modulator.

Key words: adenovirus early region 4 promoter/functional complex/purification/subunits/transcription factor

Introduction

Transcription initiation by RNA polymerase II is an important step of gene expression in eukaryotes. Initiation of transcription requires, in addition to RNA polymerase II, both general and promoter-specific transcription factors (Matsui *et al.*, 1980; Dignam *et al.*, 1983b; Fire *et al.*, 1984; Reinberg *et al.*, 1987; Reinberg and Roeder, 1987). Unique promoter specificity and regulation can be conferred by the particular composition and organization of *cis*-acting DNA sequence elements found in the promoter and enhancer regions. The promoter-specific transcription factors function by binding to these *cis* sequence elements and the rate of transcription initiation is determined by protein–DNA and protein–protein interactions (Dynan and Tjian, 1985; Serfling *et al.*, 1985; McKnight and Tjian, 1986; Maniatis *et al.*, 1987). Some of these promoter-specific factors have been identified and purified (Briggs *et al.*, 1986; Chodosh *et al.*, 1986a,b; Angel *et al.*, 1987; Lee *et al.*, 1987b; Jones *et al.*, 1987; Hai *et al.*, 1988; Yee *et al.*, 1989). Molecular cloning of the genes that encode some of these polypeptides and their functional analyses have led to the identification of the functional domains involved in DNA binding and transcription activation (Kadonaga *et al.*, 1987; Santoro *et al.*, 1988). However, the mechanisms by which these factors regulate transcription initiation are poorly understood. This is due, at least in part, to the difficulty in isolating all the factors involved in transcription from a particular promoter. Furthermore, some transcription factors have been found to form complexes with different proteins or subunits. For example, c-*jun*/AP-1 is able to form homodimers or heterodimers with itself or with c-*fos*, respectively (see Curran and Franza, 1988, and references therein). A family of CCAAT-binding proteins has been suggested to be composed of at least two different subunits (Chodosh *et al.*, 1988). Thus, when a transcription factor functions by forming a complex composed of two or more subunits, each subunit should be identified, purified and characterized to understand the mechanisms by which interactions between these subunits and other transcription factors lead to transcriptional regulation by a particular promoter.

We have identified two transcription factors, E4TF1 and E4TF3, which are involved in the transcriptional regulation of the early region 4 (E4) of adenovirus type 5 (Ad5). Both factors are bound to specific sequence elements in the E4 promoter region (Watanabe *et al.*, 1988). We have purified E4TF3 to homogeneity (Kawaguchi *et al.*, 1989) and have shown it to be identical to ATF reported by Hai *et al.* (1988). ATF has been shown to be involved in the expressions of E1A-inducible E4, E2, E3 and E1A (Lee *et al.*, 1987a; Lin and Green, 1988). This suggests that E4TF3 is involved in the common regulation of transcription from Ad E4 and other early genes, but that E4TF1 is responsible for unique regulation of transcription from the E4 promoter.

In this paper, we have purified E4TF1 to homogeneity and demonstrated that E4TF1 is composed of at least two subunits, 60 kd and 53 kd proteins. The 60 kd protein alone is able to bind to a specific DNA sequence, but does not stimulate transcription. The 53 kd protein is not able to bind to a specific DNA sequence. However, the 53 kd protein is able to interact with the 60 kd protein. This interaction is necessary for E4TF1 to function as a transcription factor.

Results

Affinity purification of E4TF1 and its transcription activity

The purification of E4TF1 from HeLa nuclear extracts is described in Materials and methods. Transcriptionally active E4TF1 was successively eluted with a flow-through fraction from a heparin–Sepharose column when loaded with 0.2 M KCl and was then eluted with 0.35 M KCl from a second DEAE–Sepharose column loaded with 0.1 M KCl (Watanabe *et al.*, 1988). Additional purification of E4TF1 was accomplished by three passages of the second column fraction through a DNA affinity column containing multiple copies of the E4TF1-binding sequence (see Materials and methods). Typical purification results are shown in Table I. It shows that the DNA affinity column is an efficient step

as expected. Fractions from the affinity purification steps were analysed by SDS−polyacrylamide gel electrophoresis (Figure 1A). Two proteins that migrated with apparent mol. wts of 60 kd and 53 kd were visualized by silver staining after three consecutive passes over the sequence-specific DNA affinity resin (lane 4). These two proteins were consistently co-purified through many trials.

In order to analyse the *in vitro* transcription activity of the purified proteins, we constructed the template shown in Figure 1C in order to eliminate recognition sites for other proteins (Watanabe *et al.*, 1988). This template gave approximately one-third the levels of transcripts as compared with the wild-type template by an *in vitro* transcription assay using a crude nuclear extract. To test transcription activity of affinity-purified E4TF1, an *in vitro* reconstituted system, basically composed of the polII, Q.03 (E4TF2) and Q.15 (E4TF3) fractions, and the template, was used as described (Watanabe *et al.*, 1988) except that E4TF3 was depleted from the Q.15 fraction using DNA affinity latex particles containing multi copies of the E4TF3-binding sequence (5′-GTGACGT-3′) as described (Kawaguchi *et al.*, 1989). The Q.15 fraction contained other general transcription factors in addition to E4TF3 (Watanabe *et al.*, 1988). Using this system, affinity-purified E4TF1 was examined for specific stimulation of transcription from the promoter used. Figure 1B shows that the addition of affinity-purified E4TF1 significantly activated the transcription. Transcripton from the promoter was stimulated ~14-fold by the addition (lane 4). No discernible change in transcription from the major late promoter (MLP), used as an internal control, was observed.

Table I. Purification of E4TF1

Fraction	Protein (mg)	Volume (ml)	Total activity (units)[a]	Specific activity (units/mg)
Nuclear extracts	280	40	–	–
Heparin−Sepharose	70	10	–	–
DEAE−Sepharose	10	20	2000	100
DNA affinity	0.003	3	300	150 000

[a]One unit is defined as the amount of protein necessary to provide 50% protection in a DNase I footprint assay.

DNA binding activity of the 60 kd protein

To determine which of the 60 kd and 53 kd proteins corresponded to E4TF1, affinity-purified E4TF1 was fractionated on a preparative SDS−polyacrylamide gel. Slices of the gel containing denatured proteins were excised, as shown in Figure 2A, and the proteins were eluted, denatured and renatured by the method of Hager and Burgess (1980). These renatured proteins were assayed for their ability to bind to the E4 promoter by gel retardation assays (Figure 2A). Band b, containing the 60 kd protein, was able to bind to the E4 promoter but yielded a DNA−protein complex that migrated faster than the complex formed with affinity-purified E4TF1 prior to separation on the preparative gel (lanes 3 and 6−8). However, the other bands a and c, used as a negative control, and d were not able to bind to the E4 promoter (lanes 2, 4 and 5). By mixing bands b and d, the mobility of the complex formed with band b shifted to that of the slow-migrating complex which had been identified as a E4TF1-specific complex (lane 1; Watanabe *et al.*, 1988). To make sure of the interaction between the 60 kd and 53 kd proteins, the dose effect of the 53 kd protein on mobility shift of the complexes was examined. Figure 2B indicates that the slow-migrating complex was formed by increasing the amounts of the 53 kd protein in the binding

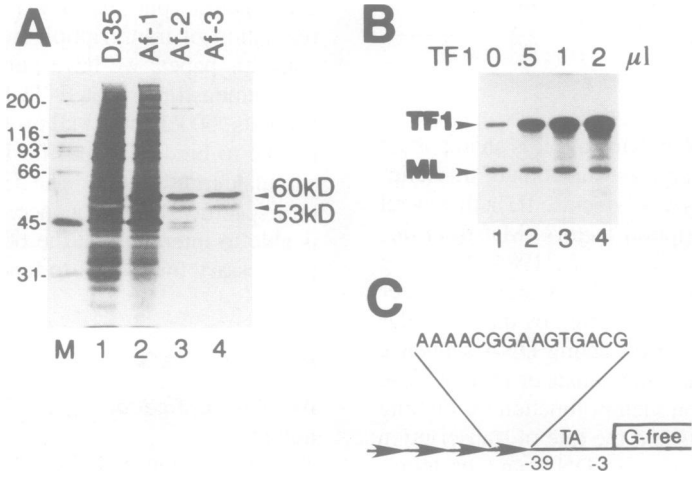

Fig. 1. Purification of E4TF1 and its *in vitro* transcription activity. (**A**) SDS−polyacrylamide gel electrophoresis of affinity-purified E4TF1 visualized by staining with silver. **Lane 1** displays the pattern of proteins obtained from pooled fractions (D.35) eluted in 0.35 M KCl through a DEAE−Sepharose column. **Lanes 2, 3** and **4** display the pattern of proteins obtained after three consecutive passes over the sequence-specific DNA affinity resin. The amounts of proteins loaded in **lanes 1** (1 μl), **2** (10 μl), **3** (10 μl) and **4** (10 μl) were equivalent to ~0.1, 1, 1 and 1 DNase I footprint units, respectively. Numbers indicate the molecular masses of standards; 200 kd myosin, 116 kd β-galactosidase, 93 kd phosphorylase, 66 kd bovine serum albumin, 45 kd ovalbumin and 31 kd carbonic anhydrase. The major 60 kd and 53 kd polypeptides in the purified E4TF1 preparation are indicated. (**B**) Transcriptional activity of affinity-purified E4TF1. Reaction conditions are described in Materials and methods. In addition, transcription reactions were supplemented with different amounts of affinity-purified E4TF1 (shown as Af-3 in A) as indicated at the top. TF1 and ML represent the 400-nucleotide length transcripts of pTF1-4(C_2AT) and the 270-nucleotide length transcripts of pdML(C_2AT), respectively. Quantitative analysis showed that transcription from the E4 promoter was stimulated 7-, 11- and 15-fold by the addition of affinity-purified E4TF1 (**lanes 2, 3** and **4**, respectively). (**C**) Schematic representation of the promoter region of pTF1-4(C_2AT) DNA used as a template. The number represents nucleotide residues from the cap site (+1) of E4 mRNA. The arrows indicate the four tandem repeated E4TF1 binding sites. The represented sequence was one of the strand of the E4TF1 binding site used.

reaction containing a constant amount of 60 kd protein. This suggests that E4TF1 contains at least the 60 kd and 53 kd proteins and that the 53 kd protein interacts with the 60 kd protein.

To confirm the direct binding of the 60 kd protein to the E4 promoter, a photochemical cross-linking protocol was used. A DNA fragment, which was the same as that used for the gel retardation assay, was body-labeled with bromodeoxyuridine and radioactive deoxycytidine according to the method described by Lin and Riggs (1974) and Chodosh *et al.* (1986a,b). The body-labeled DNA was incubated with the first affinity-purified fraction at 30°C for 30 min. Samples were then irradiated with UV light and followed by digestion with DNase I. The mol. wts of the cross-linked proteins were determined by electrophoresis on SDS–polyacrylamide gels. Figure 3 shows that the 60 kd protein directly bound to the DNA whereas the 53 kd protein did not. The result was in good agreement with that obtained by gel retardation assays (see Figure 2).

The binding site of 60 kd protein was determined by DNase I footprint analysis according to the method described by Buratowski *et al.* (1989). The binding mixtures which contained the DNA probe and both the 60 kd and 53 kd proteins were treated with DNase I before loading onto the gel. After gel electrophoresis, the two complexes and the free probe were electroblotted to NA45 paper. The paper was then autoradiographed, and the corresponding areas were cut out (Figure 4A). Labeled DNA was eluted from the paper and electrophoresed in a squencing gel. Figure 4B indicates that the same site was protected from DNase I cleavage among both complexes. This indicated that the 60 kd protein alone was able to recognize the specific sequence and that the binding specificity was not affected by interaction with the 53 kd protein.

Recognition sequence of E4TF1 and the 60 kd protein

Although Figure 4B possibly indicated that E4TF1 and the renatured 60 kd protein alone recognized the same sequence, additional analyses were carried out to confirm this issue using competition gel retardation assays (Figure 5). Complementary oligonucleotides containing a single base substitution or a 4-base substitution corresponding to the E4TF1-binding region and its flanking sequences were synthesized and used as competitors. In both cases of E4TF1 (A) and the 60 kd protein (B), the complex formations were not affected by the addition of no. 8 oligomer and were weakly affected by the additions of nos. 5, 10 and 41 as compared with the wild-type. No. 13 oligomer competed with the DNA probe as well as the wild-type. It was confirmed that the binding specificity was not changed by interaction of the 53 kd protein with the 60 kd protein. The results also suggested that the core sequence of the E4TF1 binding site was 5′-GGAAGTG-3′, although the 5′-flanking region of the core sequence exerted an influence on the binding.

The smear was observed when the 53 kd protein was absent (Figure 5B). The results suggested that the complex formed with the 60 kd protein alone was less stable than the complex formed with affinity-purified E4TF1.

Different DNA binding affinities of the two complexes

To examine whether DNA binding affinities of the two complexes, formed with the 60 kd protein in the presence or absence of the 53 kd protein were different, the sensitivity of each complex to Sarkosyl was examined. Figure 6 shows that the complex formation is reduced to 50% in the absence of the 53 kd protein by addition of 0.01% Sarkosyl whereas no discernible change is observed in the presence of the 53 kd protein. The result indicated that the 53 kd protein increased the affinity of the 60 kd for the binding site.

Protease V8 digestion

The 60 kd and 53 kd proteins were separated by SDS–polyacrylamide gel electrophoresis. The two bands containing each protein were excised from the preparative gel, the protein was partially digested *in situ* with V8 protease at the indicated amounts, and the digested polypeptides were analysed by SDS–polyacrylamide gel electrophoresis (Figure 7). The patterns of the digested polypeptides were quite different between the 60 kd and 53 kd proteins,

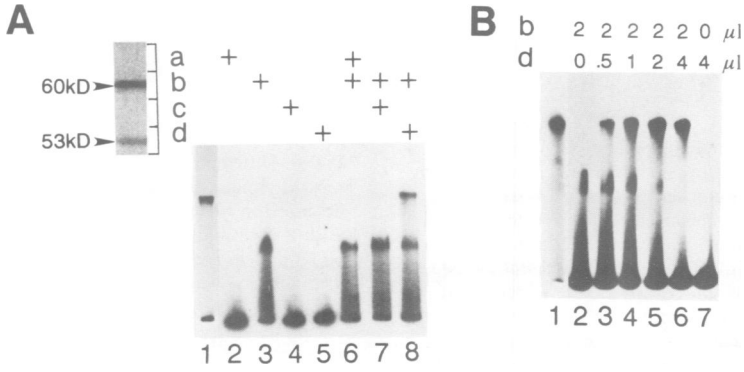

Fig. 2. DNA-binding activity in the renatured proteins isolated from SDS–polyacrylamide gel. (**A**) The subunits of affinity-purified E4TF1 (~100 ng) were separated by SDS–polyacrylamide gel electrophoresis. The materials present in **bands a, b** (containing 60 kd protein), **c** and **d** (containing 53 kd) were eluted from the gel, denatured, renatured and assayed for their DNA-binding activities using gel retardation assays as described in Materials and methods. Each material was finally suspended in 50 μl of TGE containing 0.1 M KCl and 0.1% NP40. Affinity-purified E4TF1 (0.5 μl) was used as a native source (**lane 1**). Reaction mixtures contained either an aliquot (2 μl) of the materials isolated from **bands a, b, c** and **d** or a combination as indicated at the top in the presence of 0.3 ng of a radiolabeled DNA probe including the E4 promoter region as described in Materials and methods. (**B**) Mobility of the DNA probe–60 kd protein complex was shifted by including increasing amounts of the 53 kd protein. Reaction mixtures contained the indicated amounts of the materials isolated **bands b** and **d**, corresponding to the 60 kd and 53 kd proteins, respectively. Affinity-purified E4TF1 (1 μl) was assayed as a native E4TF1 (**lane 1**).

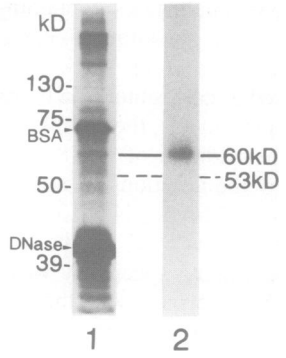

Fig. 3. Identification of the 60 kd protein by UV cross-linking. Binding reactions (50 μl) containing 3 ng of body-labeled probe (~10^8 c.p.m./μg) and 5 μl of the first affinity-purified fraction were UV irradiated for 60 min and then treated with DNase I as described in Materials and methods. Then, proteins were electrophoresed on a denaturing 10% acrylamide gel and silver stained (**lane 1**). After staining, the gel was dried and autoradiographed (**lane 2**). The positions of protein mol. wt markers, BSA, and DNase I are indicated on the left. The position of the 60 kd and 53 kd proteins are indicated on the right.

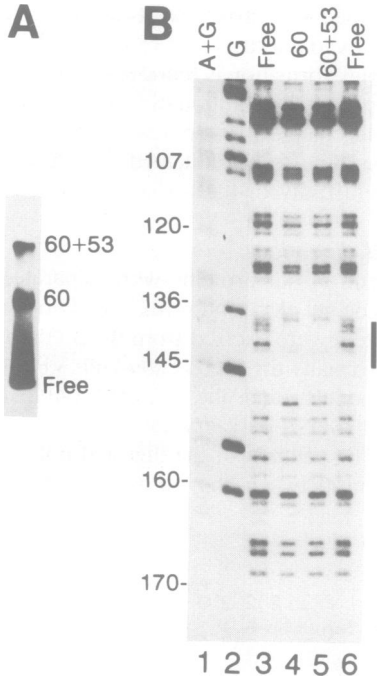

Fig. 4. Recognition site of 60 kd protein alone and 60 kd plus 53 kd protein. (**A**) Two complexes formed by the 60 kd protein alone and by the 60 kd plus the 53 kd protein were separated by gel retardation analysis. Binding reaction (12.5 μl) contained 5 μl of renatured 60 kd protein, 2 μl of renatured 53 kd protein and the end-labeled probe described in Material and methods. 60+53 and 60 represent the two complexes. Free represents the free probe. (**B**) DNase I protection pattern of DNA in the complexes. Recovery of DNA in the two complexes and free probe, and analysis by denaturing gel electrophoresis were performed as described in Materials and methods. Free DNA (**lanes 3** and **6**) showed no protection, while DNA in the complex formed by the 60 kd protein alone (**lane 4**), and DNA in the complex formed by the 60 kd plus the 53 kd protein were protected as indicated with a vertical line on the right. Numbers indicate position relative to E4 mRNA start site (+1). A+G and G are sequencing ladders of the probe fragment (**lanes 1** and **2**).

although some of the 53 kd protein was contaminating the 60 kd protein preparation. This indicated that the 53 kd protein differed from the 60 kd protein.

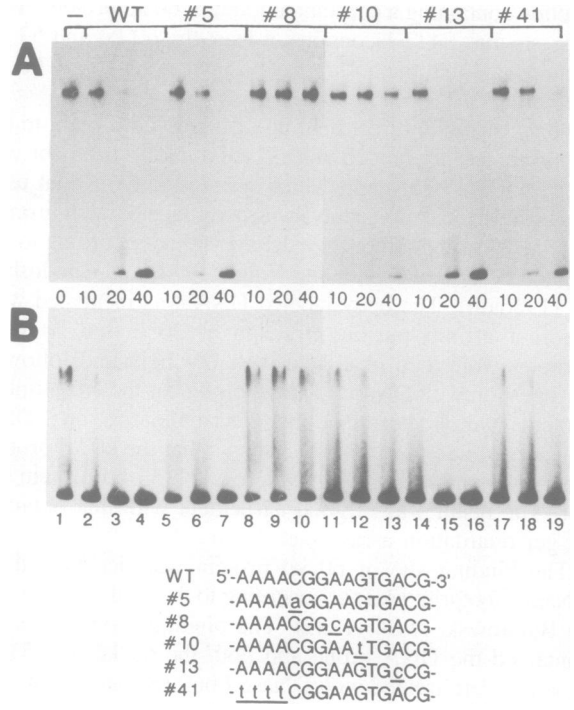

Fig. 5. Recognition sequence of E4TF1 and the 60 kd protein. Complementary oligonucleotides (one strand only shown) are indicated as WT, no.5, no.8, no.10, no.13 and no.41 below the autoradiogram were synthesized, annealed, 5'-phosphorylated and ligated to give oligomers, and were used as competitors in gel retardation assays. Substituted bases are underlined and indicated as small letters. Reaction mixtures contained 0.3 ng of DNA probe containing E4 sequences from −182 to −29, the indicated amounts of competitors and either 1 μl of affinity-purified E4TF1 (**A**) or 2 μl of the renatured 60 kd protein (**B**). The competitor DNA used and the molar excesses (10, 20 and 40) of competitor are indicated at the top. Control reaction without competitors is shown in **lane 1**.

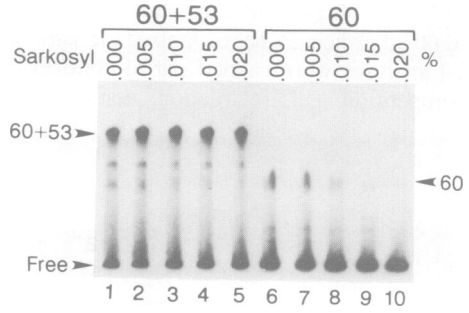

Fig. 6. Difference in the stabilities of the two complexes. Gel retardation analyses were performed according to the procedure as described in Materials and methods. Binding reactions contained either 2 μl of each renatured 60 kd and 53 kd protein or 2 μl of renatured 60 kd protein and the probe were incubated at 30°C for 30 min and then Sarkosyl was added at the indicated concentration (%) before loading onto the gel. 60+53 and 60 represent the positions of complexes formed by 60 kd plus 53 kd protein and 60 kd protein alone, respectively.

Transcription activation by the renatured 60 kd and 53 kd proteins

Transcription activities of the renatured 60 kd and 53 kd proteins were examined by reconstituted transcription assays as shown in Figure 1B and C. Figure 8A indicates that a mixture of the 60 kd and 53 kd proteins was able to specifically stimulate transcription of the template 10-fold

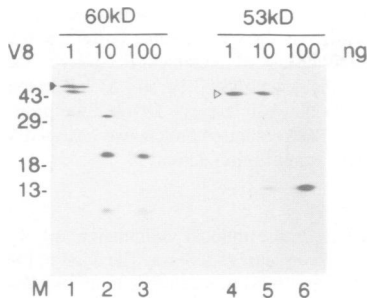

Fig. 7. Partial digestion of 60 kd and 53 kd proteins with V8 protease. The areas containing either the 60 kd or 53 kd protein were cut out from preparative SDS−polyacrylamide gels. The gel slices containing ~150 and 100 ng of the 60 kd (**lanes 1−3**) and 53 kd (**lanes 4−6**) proteins, respectively, were incubated with 1 (**lanes 1,4**), 10 (**lanes 2,5**) and 100 (**lanes 3,6**) ng of V8 protease in the sample wells and subjected to electrophoresis and staining with silver as described in Materials and methods. The 60 kd protein band was slightly contaminated with the 53 kd protein. The markers are ovalbumin (43 kd), carbonic anhydrase (29 kd), β-lactoglobulin (18 kd) and lysozyme (14 kd) from the top to the bottom. Closed and open triangles represent the position of the 60 kd and 53 kd proteins, respectively.

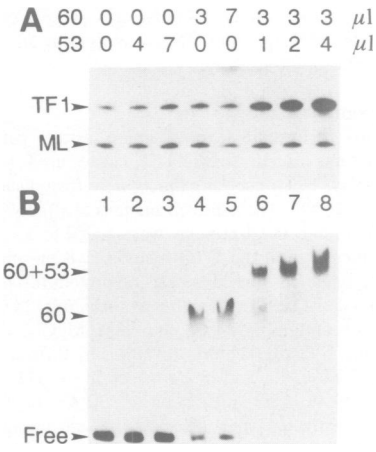

Fig. 8. Stimulation of transcription *in vitro* by 60 kd plus 53 kd protein. (**A**) Conditions and templates used in transcription reactions are described in Materials and methods. Transcription reactions (12.5 μl) were supplemented with the indicated amounts of renatured 60 kd and 53 kd proteins, shown at the top. TF1 and ML represent transcripts of pTF1-4(C_2AT) and pMLd(C_2AT), respectively. (**B**) The same amounts of the 60 kd and 53 kd proteins used in each *in vitro* transcription reaction were analysed for their DNA-binding activities by gel retardation assays (10 μl). Binding reactions contained 3 ng of DNA probe containing the sequences from −182 to −29. As a result, the molar ratio of the proteins to the DNA in the binding assay was equivalent to that in the *in vitro* transcription assay. 60+53 and 60 represent the positions of complexes formed by 60 kd plus 53 kd protein and 60 kd protein alone, respectively.

(lane 8), although no discernible change in transcription from the MLP used as an internal control was observed (lanes 6−8). However, neither the 60 kd nor the 53 kd protein alone was able to stimulate the transcription (lanes 2−5). In parallel with the transcription assays, binding activities of the same amounts of the proteins used in each transcription assay were examined by gel retardation assays (Figure 8B). This confirmed the interaction between the 60 kd and 53 kd proteins as shown in Figure 2A and B. When the effect of these renatured proteins on transcription from the E4 wild-type promoter was examined, the transcriptional activity increased ~4-fold (data not shown). These results indicated that the interaction between the 60 kd and 53 kd proteins was necessary for stimulation of transcription from the E4 promoter.

Discussion

We have purified the transcription factor E4TF1 using sequence-specific DNA affinity chromatography. Surprisingly, the purification yielded not a single polypeptide, but rather at least two polypeptides with mol. wt of 60 kd and 53 kd. These polypeptides eluted from the gel, denatured and renatured, were examined for their abilities to bind to a specific DNA sequence and to stimulate transcription *in vitro* from the E4 promoter. DNA-binding activity was found in the 60 kd protein, but not the 53 kd protein. The interaction of the 53 kd protein with the 60 kd protein increased the affinity of the 60 kd protein for the specific DNA sequence and stimulated transcription from the E4 promoter. These indicate that a transcription factor E4TF1 is composed of at least two distinct subunits. These results also suggest that the 60 kd and 53 kd proteins contribute separate functions, involved in DNA binding and transcription stimulation, respectively. Alternatively, the association of the 53 kd protein modifies the 60 kd protein, leading to exposure of the transcription-stimulating domain of the 60 kd protein. Although further analyses are necessary to conclude this issue, the 53 kd protein modulates the activity of the 60 kd protein.

For several reasons, we do not think that the 53 kd protein is a degradation product of the 60 kd protein. The partial hydrolysis of the 60 kd and 53 kd proteins with V8 protease according to the method of Kennedy *et al.* (1988) gave different patterns on SDS−polyacrylamide gel (see Figure 6). When various kinds of protease inhibitors, leupeptin (1 μg/ml), pepstatin (1 μg/ml), chymostatin (1 μg/ml) and phenylmethylsulfonyl fluoride (1 mM), were present altogether or absent during the whole purification procedure, almost the same amount of the 53 kd protein was obtained at the final purification step in both cases (data not shown). The incubation of the sample at 30°C for 1 h after the second affinity purification did not alter the ratio of these two proteins on SDS−polyacrylamide gel (data not shown). Furthermore, addition of the 53 kd protein to the 60 kd protein caused a mobility shift of the DNA probe−60 kd protein complexes (see Figure 2), and increased the stability of the complex (see Figure 6) as well as transcription stimulation (see Figure 8). If the 53 kd protein were a degradation product of the 60 kd protein it would be hard to explain these results.

Additional experimental evidence supports the assignment of E4TF1 to a complex containing the 60 kd and 53 kd proteins. E4TF1 had a native mol. wt of 100−130 kd, as determined by gel filtration (data not shown). This result suggests that E4TF1 is a heterodimer composed of the 60 kd and 53 kd proteins.

Since the mol. wt of the 53 kd protein was almost identical to that of the p53 cellular tumor antigen tightly bound to SV40 T antigen (Linzer and Levine, 1979; Lane and Crawford, 1979), we examined whether the 53 kd protein was able to react with anti-p53 monoclonal antibody in Western blotting analyses. No reaction with this antibody was detected (data not shown). Since the interaction between

the two subunits of E4TF1 was similar to that between c-*jun*/AP1 and c-*fos*, we also examined whether the 60 kd and 53 kd proteins could react with anti-c-*fos* antibody. The anti-c-*fos* antibody did not react with either the 60 kd or 53 kd proteins (data not shown).

We have already identified two transcription factors, E4TF1 and E4TF3, which bind to specific sequence elements in the Ad E4 promoter. E4TF3 has been purified to homogeneity from HeLa nuclear extracts by using affinity latex particles developed by us and has been shown to be identical to ATF (Kawaguchi *et al.*, 1989). The binding sites of ATF are commonly present in other early promoters. It has been indicated that ATF is involved in their transcriptional regulation, suggesting that ATF is concerned with the common regulation of Ad early gene expression. However, E4TF1 or E4TF1-like factors responsible for transcriptional regulation have not been demonstrated in other Ad early promoters. Therefore, the results presented in this paper suggest that E4TF1 has an important role in the unique regulation of E4 gene expression. The interaction between 60 kd and 53 kd proteins is a key to understanding the unique regulation of transcription from the E4 promoter. We are now making antibodies against these two proteins and are trying to isolate cDNA clones of these protein genes. We are also analysing the interaction of these E4TF1 subunits with general transcription factors, ATF and viral E1A and E2A gene products, to elucidate the mechanisms of transcriptional regulation of the E4 gene by multiple cellular and viral transcriptional factors.

Materials and methods

Preparation of a sequence-specific DNA affinity resin

Two complementary oligonucleotides, 5′-AAAACGGAAGTGACG-3′ and 5′-TTTTCGTCACTTCCG-3′, were synthesized on a DNA synthesizer 380B (ABI). They were 5′-phosphorylated, annealed and ligated to give oligomers that included core DNA sequences of the E4TF1 binding site (see Figure 7 and Watanabe *et al.*, 1988). The DNA oligomers were covalently attached to Sepharose CL-2B by the method of Kadonaga and Tjian (1986).

Extracts and purification of E4TF1

HeLa nuclear extracts were prepared according to Dignam *et al.* (1983a). Chromatography of the HeLa nuclear extract to generate E4TF1, Q.03 (E4TF2), Q.15 (E4TF3) and polII fractions was performed as described (Watanabe *et al.*, 1988). E4TF1 was eluted with a flow-through fraction from a heparin–Sepharose column (Pharmacia) when loaded with HGME buffer containing 0.2 M KCl. The eluted fractions were pooled and ammonium sulfate was added to 60% saturation. The precipitate was collected by centrifugation, dialysed against TGME buffer containing 0.1 M KCl and applied to a DEAE–Sepharose column equilibrated with TGME buffer containing 0.1 M KCl. E4TF1 was eluted with TGME buffer containing 0.35 M KCl through the column. This 0.35 M KCl fraction was dialysed against TGE buffer containing 0.1 M KCl and 0.1% Nonidet P40 (NP40) for 4 h and loaded onto an E4TF1-specific affinity column. The column was washed extensively with TGE buffer containing 0.1 M KCl and 0.1% NP40. E4TF1 was eluted with TGE buffer containing 1.0 M KCl and 0.1% NP40. The 1.0 M KCl was directly diluted to 0.1 M KCl by adding KCl-free TGE containing 0.1% NP40 and then the same purification procedure was repeated twice. Two, 1 and 0.5 μg/ml of poly(dI-dC)(dI-dC) were present during the first, second and third passes over the affinity resin. The protein concentration at the final step was roughly estimated by the density of the silver-stained band in SDS–polyacrylamide gels. The affinity-purified E4TF1 was assayed for specific binding activity by gel retardation analysis and further fractionated by SDS–polyacrylamide gel electrophoresis.

Approximately 100 ng of affinity-purified E4TF1 (100 μl) was usually applied to a preparative SDS–polyacrylamide gel. Two subunits of E4TF1 were eluted from the gel, acetone-precipitated, denatured and renatured according to Hager and Burgess (1986), except that each denatured sample was suspended in 50 μl of TGE containing 0.05 M KCl and 0.1% NP40 and renatured by dialysis against the same buffer for 6 h to remove the guanidium hydrochloride instead of dilution. Recovery of the binding activity from a mixture of the renatured 60 kd and 53 kd proteins was between 30 and 60% when roughly estimated by DNase I footprint analysis and gel retardation analysis. Each renatured protein was analysed for DNA binding activity and *in vitro* transcription activity.

Plasmids

In order to detect transcriptional stimulation of E4TF1, plasmid pTF1-4(C_2AT) was constructed as follows: The *Sma*I of pML(C_2AT) DNA (Sawadogo and Roeder, 1985) was converted into the *Hin*dIII site and the *Sma*I–*Hin*dIII polylinker region was deleted to remove the *Bam*HI site. Then, the *Eco*RI site of the plasmid was changed to the *Bam*HI site. The resulting *Bam*HI–*Sst*I site of this plasmid DNA was inserted by the fragment containing the sequence from −39 to −3 relative to the cap site (+1) of the E4 mRNA to give pE4TA(C_2AT) DNA. Complementary oligonucleotides used for the sequence-specific DNA affinity column were annealed, 5′-phosphorylated and ligated to give oligomers. The ends of the oligomers were filled up, ligated with *Bam*HI linkers, and inserted into the *Bam*HI site of pE4TA(C_2AT). After cloning and sequencing the inserted DNA fragments, a plasmid in which four E4TF1 binding sites were tandemly repeated in the same orientation as the wild-type was selected and used as plasmid pTF1-4(C_2AT).

Plasmid pML(C_2AT) DNA (Sawadogo and Roder, 1985) was digested with *Sma*I, treated with *Bal*31, filled up and ligated to give plasmid pMLd(C_2AT). Deletion of ~130 bp from the 3′ end of the G-free cassette resulted in a transcript 270 nucleotides in length. It was possible to distinguish between the ML transcripts and the E4 transcripts, 400 nucleotides in length, by their sizes. This pMLd(C_2AT) DNA was used as an internal control for the *in vitro* transcription assay.

In vitro transcription assays

For the *in vitro* reconstituted transcription assay, partially purified transcription factors, except for E4TF1, were used to obtain basal transcription activity. HeLa nuclear extracts were fractionated as described by Watanabe *et al.* (1988). The indicated amounts of affinity-purified E4TF1 or renatured 60 kd and 53 kd proteins were added to a reaction mixture (12.5 μl) containing 2 μl of the Q.03 fraction (1.8 mg protein/ml), 2 μl of the E4TF3-depleted Q.15 fraction (1.2 mg protein/ml) described in the text, 2 μl of the polII or D.225 fraction (3 mg protein/ml), 25 mM Tris–HCl, pH 7.9, 9.6% glycerol, 50 mM KCl, 6 mM $MgCl_2$, 0.5 mM DTT, 0.6 mM ATP and CTP, 0.02 mM [α-^{32}P]UTP, 0.02 mM 3′-*o*-methyl GTP, 20 units RNase T1 and 5 μg/ml each of pTF1-4(C_2AT) and pdML(C_2AT) DNAs. After incubation at 30°C for 60 min, RNA transcripts were extracted and analysed by electrophoresis on 6% polyacrylamide/7 M urea gel. The intensity of the bands was quantitated by tracing the autoradiographs with an ATAGO densitometer. The amount of the E4 band was calculated relative to the MLP band.

Gel retardation assay

Binding reactions and gel electrophoresis were performed as described by Watanabe *et al.* (1988), except that 3 ng of the DNA probe was usually used, and poly(dI-dC)(dI-dC) was removed from the reaction mixture. DNA probes containing the E4 promoter were prepared from plasmid pUCE4-20 that contained the sequence from −29 to −182 relative to the cap site of E4 mRNA (Watanabe *et al.*, 1988). The plasmid was digested with *Eco*RI and *Hin*dIII and end-labeled with Klenow fragment and [α-^{32}P]dATP. The 204-bp fragments were purified by agarose gel electrophoresis. The binding reaction (12.5 μl) contained 12% glycerol, 40–60 mM KCl, 30 mM Tris–HCl, pH 7.9, 0.6 mM EDTA, 3 ng of the probe and the indicated amounts of affinity-purified E4TF1, renatured 60 kd protein, or renatured 60 kd plus 53 kd protein.

DNase I protection analysis

DNA footprint probes were prepared by filling in the 3′ end with [α-^{32}P]dATP and Klenow fragment at the *Hin*dIII sites of pUCE4-20. After digestion with *Eco*RI, the 204-bp DNA fragments were purified on an agarose gel. DNase I footprint analysis was carried out according to the method described by Buratowski *et al.* (1989). Binding reactions were performed with the same volumes as in the gel retardation assays. Reactions were incubated at 30°C for 30 min and then loaded on to the native gels immediately after DNase I treatment. After gel electrophoresis, the complexes and free DNA probes were electroblotted to NA45 paper (Schleicher and Schull). The paper was then autoradiographed, and the area corresponding to the complexes and free DNA probes were cut out. Labeled DNA was eluted from the paper and electrophoresed in a sequencing gel.

UV cross-linking

UV cross-linking reactions were performed according to Chodosh *et al.* (1986a,b). pUCE4-20 DNA was digested with *Eco*RI, and partially digested with the 3′→5′ exonuclease activity of T4 polymerase in the absence of deoxynucleoside triphosphates to remove ~200 nucleotides from the *Eco*RI-cut end. The DNA was then resynthesized with 50 μM dATP, dGTP, 5′-bromo-2′-deoxyuridine triphosphate and 5 μM [α-^{32}P]dCTP by the method of O'Farrell *et al.* (1980) and digested with *Hin*dIII. The 204-bp DNA fragment containing the sequences from −29 to −182 relative to the E4 mRNA start site was purified by agarose gel electrophoresis. In 50 μl of reaction mixture, 3 ng of the body-labeled DNA was mixed with 5 μl of the first affinity-purified fraction for 10 min at 4°C in the presence of 20 μg/ml bovine serum albumin and UV irradiated for 60 min. Then, 80 μg/ml DNase I was added to the mixture. After incubation at 37°C for 30 min, SDS sample buffer was added to the mixture and boiled for 3 min. Half of the sample was loaded onto SDS−polyacrylamide gel. After gel electrophoresis, the gel was stained with silver, dried and exposed to X-ray film.

Protease V8 digestion

Materials isolated from preparative acrylamide gels were digested *in situ* using *Staphylococcus aureus* V8 protease as described by Kennedy *et al.* (1988). The gel slices containing the 60 kd and 53 kd proteins were cut out of the preparative gels, soaked in deionized water for 15 min and then loaded onto a 15% acrylamide gel as an acrylamide cube. After loading, the sample well was filled with the indicated amounts of V8 protease (Sigma) in buffer containing 20% glycerol, 0.05 M Tris−HCl, pH 6.8, 0.2% bromophenol blue and 0.1% SDS. A 1:1 (v/v) ratio of gel slice to protease buffer was used. The acrylamide/protein/enzyme mixture was allowed to incubate in the well for 15 min at room temperature and then run to the stacker (separating gel interface at a low constant voltage (20−30 V). At this point the current was turned off for 1 h, to allow the enzyme to digest the protein. After digestion, electrophoresis was completed and the gel was stained with silver.

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